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Temporal correlation between neuronal tail ganglion activity and locomotion in the leech, *Hirudo medicinalis*

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ABSTRACT Intracellular and extracellular recordings were performed in the posterior ventral nerve cord of restrained crawling preparations of the medicinal leech, *Hirudo medicinalis*. Short-latency neuronal activities in the tail ganglion nerves correlated with different phases of crawling behavior. Eight neurons with characteristic activation patterns during crawling were identified morphologically and physiologically in the tail ganglia of 23 preparations. The axons of four of these neurons projected through posterior tail brain nerves; four ascending interneurons had projections in the connectives or in Faivre's nerve. These interneurons are suitable candidates for carrying information between the front end and the tail end of the animal to coordinate the behavioral components during a crawling step.

KEY WORDS: *Hirudo medicinalis*; leech; locomotion; motor pattern; intracellular recordings

Introduction

In the medicinal leech, the control of simple behaviors such as the local shortening reflex or the local bending reflex occurs mainly locally, on the level of individual midbody segments. In contrast, the circuitry for swimming consists of several hierarchically organized neuronal levels (Brodgheuer and Friesen, 1986a) which are located in all body parts. Segmental pattern generators (CPGs) which drive the motor output (Friesen, 1989) are regulated by descending pathways from the head brain. Several descending interneurons have swim-activating or swim-inactivating effects (Brodgheuer and Burns, 1995), that is, they either trigger swimming (Brodgheuer and Friesen, 1986b, c, d) or they terminate an ongoing swim episode (O'Gara and Friesen, 1995). Moreover, excitatory drive from the tail brain helps both to lower the threshold for swim initiation and to prolong swim episodes (Brodgheuer *et al.*, 1993, 1995). The neuronal circuitry for crawling behavior has only recently begun to be elucidated (Baader and Kristan, 1992, 1995; Eisenhart *et al.*, 1995; Baader, 1997). Behavioral observations in leeches on which surgical manipulations of the nervous system had been performed, suggested that both terminal brains were important to produce complete crawling cycles (Gray *et al.*, 1938; Baader and Kristan, 1995). Therefore, both locomotor behaviors, swimming and crawling, seem to need additional regulatory drive deriving from the head ganglion and from the tail ganglion. This paper describes neurons in the tail ganglion that participate in crawling behavior. Extracellular recordings from tail brain nerves were

performed during crawling. Neural activity was measured with short latencies to behavioral events occurring at the front end of the animal. Eight neurons in the tail brain are described morphologically and physiologically. Their spike activities were characteristically modulated during different phases of crawling.

Methods

Animals and preparation

Adult leeches (*Hirudo medicinalis*) were obtained from commercial suppliers (Leeches USA, Westbury, NY; Moser Blutegelhandel, Schorndorf, Germany), and kept in laboratory colonies at 12 °C. Animals weighing between 2.5 and 3.5 g were anesthetized in a solution of 8% ethanol in leech saline (Muller *et al.*, 1981), and pinned down with insect pins dorsal side up on a sylgard-covered dish filled with leech saline. To dissect the posterior part of the animal containing the tail ganglion, an incision was made through the body wall along the dorsal midline between segment 18 and the tail end. After the gut and overlying tissue had been removed, the ventral blood sinus around the ganglia chain was opened. The blood sinus around the tail brain tightly adheres to the ganglion sheath. It was carefully pulled off the ganglion sheath with a sharp forceps before the sheath was removed from only those neuromeres where intracellular recordings were performed. The tail sucker was dissected leaving some connective tissue around the tail nerves intact, which was later used to fixate the tail brain. All nerves from ganglion 18 through the tail brain were cut at their

distal ends before the posterior body wall from segment 19 to the tail sucker was removed. Depending on the type of experiment, those nerves from which extracellular recordings were to be obtained were left at sufficient lengths.

Anatomy of the posterior nervous system

The gross morphology of the posterior ventral nerve cord is illustrated in Fig. 1. Six animals were stained by injecting Methylene Blue (3% in saline) into the posterior body cavity. Animals were then anesthetized, the body wall was opened, and after rinsing the body cavity several times with saline, the individual nerves were traced through a dissection microscope. Fig. 1 shows the dorsal aspects of the last three midbody ganglia (M19–M21) and of the tail ganglion, which are linked by a pair of connectives and a single Faivre's nerve. In *Hirudo*, each connective contains about 2800 axons, and 97 axons run in the Faivre's nerve (Wilkinson and Coggeshall, 1975). The tail ganglion is divided into seven (caudal) neuromeres (C1–C7). Two lateral roots emerge from each midbody ganglion and divide into several branches. Using the description of Ort *et al.* (1974), the dorsal (DP) and the posterior (PP) branch of the posterior nerve could be

readily re-identified in M19 to M21. However, in contrast to anterior body segments, the anterior root (A) in M20 and M21 was often in close association to the PP nerve. Each neuromere of the tail brain has a pair of nerves on each side that run into the periphery towards the musculature of the tail sucker (grey circle designates sucker outline in Fig. 1). The posterior nerve in each neuromere was usually slightly thicker than the anterior one. By analogy to the midbody ganglia these nerves were named according to their position in the corresponding tail neuromere (for example, CP1, for the posterior nerve in the first caudal neuromere, and so on).

Experimental setup

Modified versions of a previously described crawling apparatus (Baader and Kristan, 1992) were used for extracellular and intracellular recordings from the tail ganglion during crawling behavior. Animals were tethered in a saline-filled tank that contained a floating ball, and an extended horizontal platform with a small glass window in the center (Fig. 2). A circular ring of wax was formed around the glass window. After the dissection of the animal, the body wall of denervated segment 18 was pinned down to the wax base with insect pins that were inserted through the musculature. The tail ganglion was centered above the glass window and fixed with insect pins which penetrated the remaining tissue surrounding the tail nerves. The ganglion was illuminated with a halogen light source

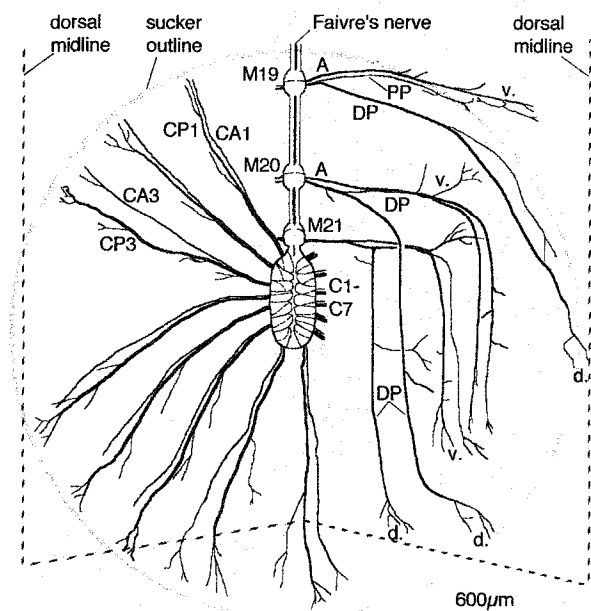


Fig. 1. Dorsal aspect of the posterior ventral nerve cord of the leech, *Hirudo medicinalis*. Schematic drawing of the last three midbody ganglia (M19–M21) and of the tail ganglion. The latter consists of seven (caudal) neuromeres (C1–C7). For clarity, only the right nerves of the midbody ganglia and the left and posterior nerves of the tail ganglion are drawn. The dorsal midline of the body wall is indicated by dashed lines, the underlying sucker is outlined by the grey circle. A = anterior, DP = dorsal posterior, and PP = posterior root of the midbody ganglia; CA = anterior and CP = posterior root of the tail brain. d. = dorsal, v. = ventral.

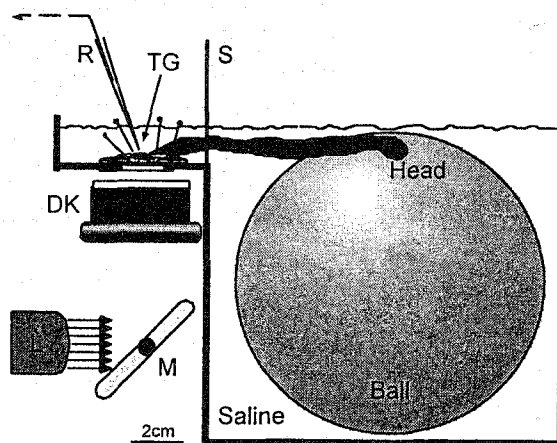


Fig. 2. Apparatus to record neuronal activity in the tail brain during crawling behavior. The animal is suspended in a saline-filled tank which contains a ball and a horizontal platform. While its tail end is tethered to the stage it can produce extensions and contractions, using the floating ball as substrate for its front sucker. The tail ganglion (TG) is exposed, and centered in the middle of a small glass plate. It is illuminated by a halogen light source (L) through a mirror (M) and a dark field condensor (DK). A metal screen (S) prevents the animal from touching the recording electrode (R).

and a darkfield condensor (MBL 12010, Nikon) which was centered under the horizontal platform below the glass window. For some experiments leeches were pinned down on a small wax covered plate (15 × 30 mm) (Fig. 3). The tail ganglion was fixed to the wax plate dorsal or ventral side up with insect pins using the connective tissue around the nerves. A small metal spoon which had the shape of the tail ganglion provided additional stability for the ganglion. Instead of using the darkfield illumination, individual cell bodies were visualized by illuminating the tail ganglion with a small light guide from above.

morphologically, electrodes were filled with 5% of the dye Lucifer Yellow (Stewart, 1978) and with 1 M LiCl. After the iontophoretical application of the dye the ganglia were processed histologically using conventional techniques. The neurons were drawn from whole mounts with a fluorescence compound microscope and *camera lucida*. Extracellular recordings were obtained either *en passant* from the connectives between M19 and M20 or from the cut ends of peripheral nerves using suction electrodes. Electrophysiological data were displayed on the screen of a storage oscilloscope and recorded on a tape recorder. A video

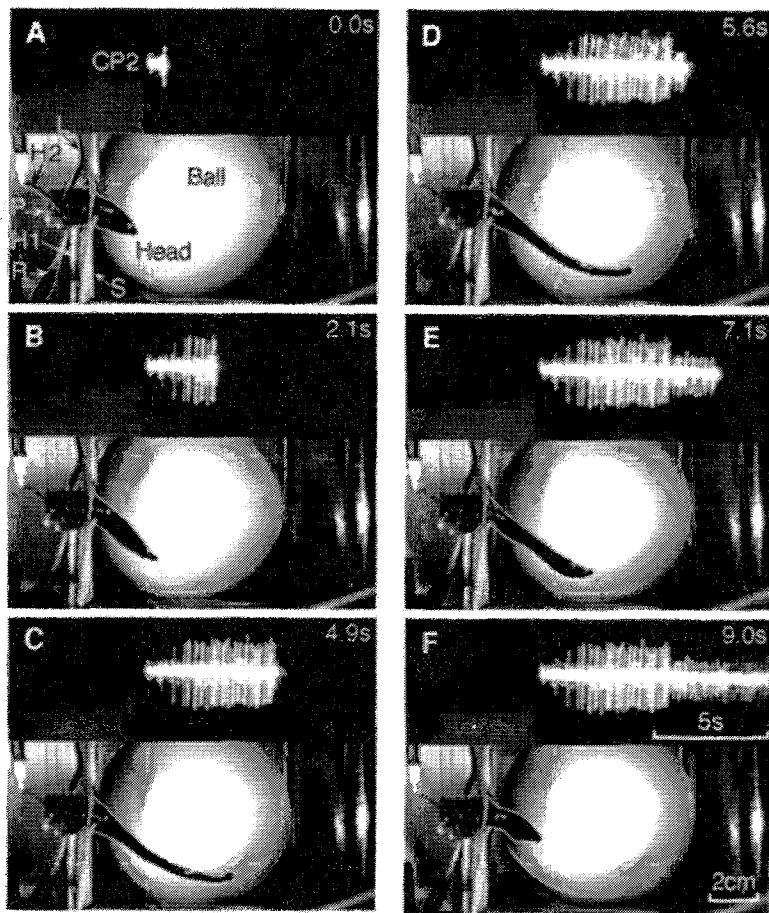


Fig. 3. Leech crawling behavior together with the extracellular recording from tail brain nerve CP2. Apparatus is slightly different from the one shown in Fig. 2, see Methods. Video images were taken from the top at six events during a step at the times indicated. Some objects were later outlined by white hair lines to improve visibility. The animal's posterior end is tethered to a square platform (P) at the end of a stainless steel rod (H1). The ganglion is additionally supported by a metal spoon (H2) and illuminated by a small light guide (L). A metal screen (S) prevents the animal from touching the recording electrode (R). The animal initiated the step by lifting its front sucker from the ball (A) and extending its body (B). Then it re-attached with its front sucker to the substrate which terminated the elongation phase (C, D). The animal shortened with a wave of segmental contractions travelling along its body from anterior to posterior (D–F), while its front sucker remained attached to the ball. After full contraction (F) it released its front sucker to initiate a new step. During the crawling cycle two main units could be distinguished in the extracellular recording: a large unit was active during the elongation phase (A–D), and a small unit fired during the contraction phase (D–F).

Intracellular recordings were obtained with glass microelectrodes filled with 3 M KAc, which had final resistances of 20–60 M Ω . To characterize neurons

camera system (PV535, Panasonic) recorded the image of the oscilloscope screen together with the behavior of the animal. Additionally, four behavioral events

during each crawling step (the onset and offset of the elongation phase, 50% and 100% contraction) were recorded with an electrical event recorder on tape together with the physiological data.

Evaluation of the physiological data

The technique for correlating neuronal events with behavior has been described previously (Baader, 1997). In brief, responses of individual neurons during crawling episodes were evaluated by measuring their firing patterns during six behavioral events at each crawling step: during the elongation from front sucker release (fs-) to one second later, one second before the re-attachment of the front sucker (fs+), and during one second at the mid-point between these two events; during the contraction for one second after fs+ (beginning of contraction), during a one second period just before maximum contraction had occurred (end of contraction), and during one second at the mid-point of these events. The inter-step intervals during crawling episodes of some animals were 500 ms and longer. In these cases neuron firing frequencies at rest were evaluated as well. An additional event was calculated when the apparent minimum or maximum of the crawling-correlated firing patterns occurred at neither of the behavioral fix-points mentioned above. The mean firing frequencies during several steps at each of the behavioral events produced the activity profiles for the individual neurons. The attachment of the front sucker was found to consist of several behavioral elements, and could take more than 100 ms. The sucker becomes bowl-shaped shortly before its first physical contact with the substrate, and before the actual sucking occurs. Therefore, for the evaluation of the video images and for the measurements shown in Fig. 4 the moment in which the sucker became bowl shaped was taken as the onset of the attachment (fs+), and as the end of the elongation phase.

Results

With their rear end tethered in the crawling apparatus, the animals produced complete crawling episodes. Fig. 3 shows video images of a typical crawling step, together with the extracellular recording of tail brain nerve CP2. The images were taken at six time points during the step. The animal initiated a step by lifting its head sucker off the ball (Fig. 3A) and extending its body (Fig. 3B). A large unit in the extracellular recording was activated shortly before the beginning of the elongation phase (see also Fig. 4). This unit remained active throughout the elongation, and until

the front sucker re-attached to the substrate (Fig. 3C). With the onset of the contraction phase (Fig. 3D) the large unit ceased to fire, and a tonic unit of smaller amplitude became active instead. The animal shortened with its front sucker attached to the ball (Fig. 3E, F). The smaller extracellular unit remained excited throughout the contraction phase (Fig. 3F). In extracellular recordings from posterior tail brain nerves in 15 animals these two units could easily be distinguished by their different amplitudes, and by their distinct activation pattern during crawling. Both units were quiet in non-moving animals, and they were found in all posterior tail nerves. During crawling, their activity patterns were strongly correlated with the elongation and contraction phase (Fig. 4A), and their firing frequencies were rather independent from the actual length of the animal during crawling. Fig. 4B quantifies the temporal relationship between the occurrence of the large unit and the elongation phase of the animal. In the CP1-recording, the first action potential occurred 125 ± 57 ms (mean \pm S.E.M., $n = 23$ steps) before the first visible forward movement of the head of the animal, i.e. the initiation of the elongation phase. The spike train ended 266 ± 51 ms after the front sucker became bowl-shaped, i.e. the beginning of the front sucker attachment. The small unit was turned on immediately after the large unit ceased to fire, and when the animal began to contract. The large unit was not only active during the elongation

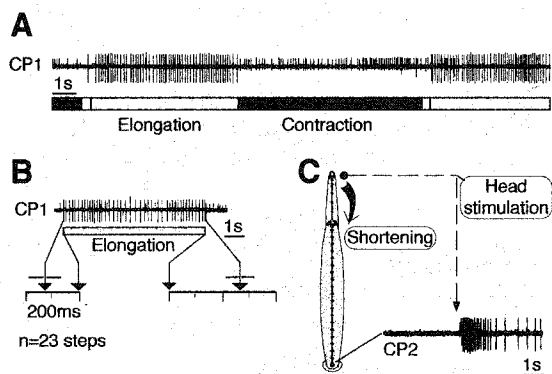


Fig. 4. Temporal correlation between neural activities in tail brain nerves and behavior. A. Recording of nerve CP1 (upper trace) together with crawling steps (lower trace). The behavioral components (Elongation, Contraction, Pause) are marked by the rectangles shaded in grey and white. Two units are visible in the recording (see also Fig. 3); a large unit was active during elongations and a small unit was visible during contractions. B. Temporal analysis of the large unit of CP1 during 23 steps. The cell began to fire before the onset of elongations, and continued until the elongations were terminated. The arrows show the mean delays with the horizontal bars indicating \pm S.E.M. C. Response of the large unit in a CP2-recording to mechanically touching the tip of the head of the animal which elicited the whole body shortening reflex.

phase of crawling, but also when the animal's head was mechanically stimulated by a brief stroke with a glass rod (Fig. 4C) to induce a shortening reflex (Kristan, 1982). In this case, the first response in the CP2-nerve of the tail end occurred with a delay of 280 ± 9.6 ms (mean \pm SEM, $n = 6$ stimulations) after the first visible withdrawal of the front end.

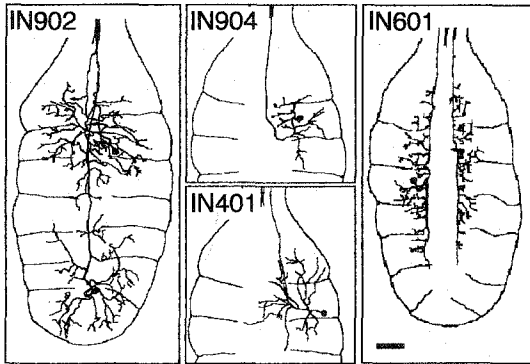


Fig. 5. Reconstructions of four ascending interneurons on the ventral aspect of the tail ganglion which were activated during crawling. Camera lucida drawings of the Lucifer Yellow-stained neurons. The examples shown for IN601 and IN902 were stained in different preparations. IN902 and IN904 projected into the Faivre's nerve, IN401 and IN601 ascended through the connectives. Bar 100 μ m.

Firing patterns of tail ganglion neurons

Intracellular recordings in the tail brain were performed to trace neurons with characteristic activation patterns during crawling behavior. Four ascending interneurons on the dorsal aspect of the tail brain were identified morphologically and physiologically in a total of 14 preparations (Figs. 5, 6). IN401 and IN601 (found on both sides of the second and third neuromere) ascended through the connectives (Fig. 5). The axons of IN902 and IN904 projected into the Faivre's nerve. IN902 was recorded in the third and seventh neuromere. It had bilaterally distributed arborizations within the ganglion while those of IN904, IN401 and IN601 remained mainly within the ipsilateral half of the ganglion. The branches of all neurons extended into neighboring neuromeres. During crawling, IN902 was increasingly active at the late contraction phase and it had its firing maximum at the beginning of the elongation phase (Fig. 6A, B). The responses of the other three neurons occurred with opposite phase lags: all three were maximally excited at the end of the contraction phase and least active during elongations. In three of the four interneurons their ascending axons were traced with extracellular suction electrodes in the connectives between ganglion 19 and 20 (Fig. 6C). Corresponding action potentials in the connectives were recorded with constant delays of 4 ms to 8 ms. Three of these interneurons (IN401, IN601 and IN904) could be

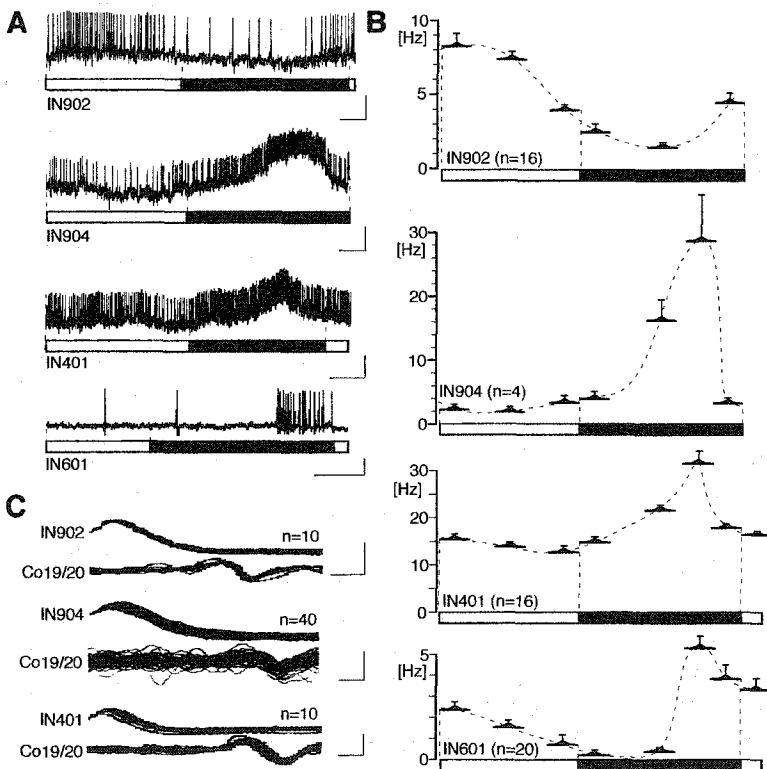


Fig. 6. Intracellularly recorded responses of the ascending interneurons shown in Fig. 5. A. Representative activity patterns of each cell during a single step. Bars 2 s, 4 mV. B. Mean activity profiles (\pm S.E.M.) during the number of steps indicated. C. Simultaneous intracellular recordings from the cell bodies in the tail ganglion and extracellular suction electrode recordings from the connectives/Faivre's nerve between ganglion 19 and 20. Bars 1 ms, 4 mV. Superimposed recordings with the number of trials indicated. For color coding of the bars see Fig. 4A.

recorded when the animals started to swim but no change in activity during the swim bouts was observed (data not shown). Additionally, the tip of the head was stimulated with a glass rod to induce the shortening reflex. There was no change in activity in IN401 and IN601, and a slight increase in excitation in IN902 and IN904 (data not shown).

Four neurons with axons leaving the ganglion through the posterior nerves were identified in nine preparations (Fig. 7A). N31 was most strongly excited at the beginning of the elongation phase (Fig. 7B, C). This excitation gradually decreased to a minimum when the animal contracted. The other three cells, N32, N33 and N41, were only weakly excited during the elongation phase and received stronger drive during contractions.

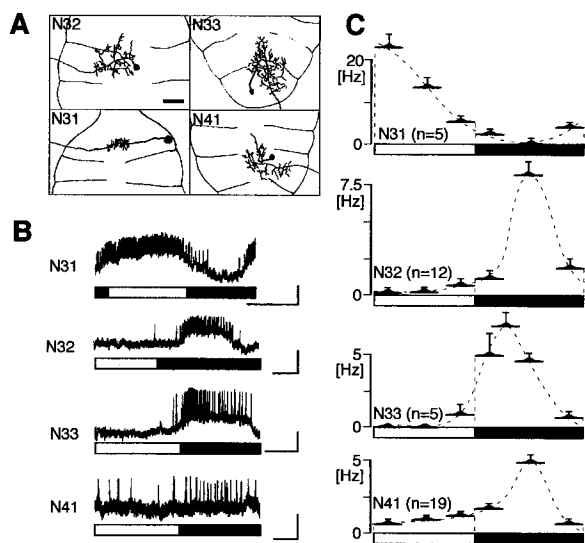


Fig. 7. Morphological and physiological characteristics of four tail ganglion neurons which had axons projecting through the posterior nerves. Bar 100 μ m. A. Reconstructions of the neurons on the ventral side of the tail ganglion which were stained with Lucifer Yellow. Bar 100 μ m. B. Neuron responses during single crawling steps. C. Averaged firing frequencies (\pm S.E.M.) during the number of crawling cycles indicated. Bar 2 s, 4 mV. For color coding of the bars see Fig. 4A.

Discussion

Restrained leeches performed repeated crawling episodes in the apparatus although their tail suckers were tethered to the platform, and their posterior ganglia were exposed and denervated (Fig. 3). For normally crawling animals, the attachment and the release of the rear sucker are important components of the crawling cycle. A failure of the rear sucker to release would prevent the animal from pulling forward. Likewise, the animal normally releases its front sucker to begin a new step after it has firmly attached its rear sucker. But the experiments with tethered leeches

show that tail sucker activities are not necessary components for the principal production of rhythmic crawling movements. If tail sucker activity is prevented, the animals nevertheless produce continuous crawling cycles. These findings support previous results where animals which had their tail suckers covered to prevent attachment and release (Baader and Kristan, 1995) or which had denervated suckers (Gray *et al.*, 1938) nevertheless produced cyclic crawling movements.

Neuronal activity recorded extracellularly in tail brain nerves and intracellularly from various neurons occurred temporally linked to the crawling rhythm. In posterior tail ganglion nerves, two different units were easily distinguishable. Although it was not possible to identify the two units morphologically, their firing patterns were clearly linked to behavior. The activation of the larger unit occurred about 125 ms before the beginning of elongation. This activity was correlated to the initiation of the elongation and not to the end of the contraction of the preceding step since it was also observed at the beginning of new crawling sequences with no immediate preceding contractions. Can this delay between neural activity and the onset of the extension phase help to suggest possible sites for the generation of the crawling rhythm? Previous dissection studies have suggested that crawling is under the control of the head brain and of the tail brain (Baader and Kristan, 1995). If it is generated in the head brain, a signal must be initiated in the head, travel down the ventral nerve cord and cause the observed excitation in tail ganglion nerves, about 125 ms before the beginning of the first elongation is actually observed at the head end. The existence of a principal pathway from the head to the tail is documented by the responses in CP-nerves after mechanical head stimulation (Fig. 4C), but the significance of this pathway for crawling has yet to be shown. On the other hand, if a step is initiated in the tail brain, an ascending signal must travel up to the head and trigger the onset of elongation in the tip of the head, with the subsequent extension of the body. The necessary delay for such a signal travelling through the ventral nerve cord could well be in the range of what was measured between the onset of the large unit in the CP-nerves and the first observed extension of the tip of the head (≈ 125 ms).

The activity patterns of the ascending interneurons and their extracellular recordings from the connectives confirmed a possible importance of the tail ganglion for crawling. The delays of the action potentials of IN902, IN904 and IN401 in the connectives at the level of ganglion 19 were in the range of 4 to 8 ms. Under the assumption that the axons of these neurons

project further along the ventral nerve cord, their signals could theoretically arrive in the head brain after about 50 to 100 ms. These values compare well to those of another identified interneuron, the S-cell (Peterson, 1984). This cell has the largest diameter axon in the ventral nerve cord and is, with an inter-segmental travel time of 2 ms, the fastest neuron in the leech nervous system. An action potential of this cell generated at one end of the animal would arrive at the other end after around 40 to 50 ms. Interestingly, the S-cell can also produce bursts during the contraction phase of crawling (Baader, 1997).

The other group of neurons excited during crawling were cells N31–N33 and N41. Their morphology suggests that they are motor neurons. However, the identification of motor neurons requires simultaneous intracellular recordings from the neuron and the muscle fiber (Stuart, 1970). The type of preparation used here did not allow muscle fiber recordings, nor was it possible to trigger correlated muscle twitches upon intracellular stimulation of the neuron, an alternative though less conclusive method.

In conclusion, the temporally organized activation of the two suckers during crawling requires fast pathways between the head and the tail. The recorded correlation between extracellularly recorded activity in the tail ganglion and the movement of the tip of the head indicated indirectly the presence of such fast connections. Additionally, the ascending interneurons had short conduction times in the connectives posterior to ganglion 19. Moreover, they were strongly modulated during crawling and none of these neurons was found to be active during swimming. These data emphasize the important role of the tail ganglion during crawling and make the described interneurons good candidates for the communication between the two ends of the animal during this locomotory behavior.

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